Transcribed from Record Received 12/27/57
Memo from Joshua Lederberg

Madison, Wisconsin
December 22, 1957

Dear Joe:

This is a memorandum of the topic we discussed yesterday designed mainly to refresh my own memory. If convenient for you, I would very much appreciate having a typed copy in due course.

If my memory serves, our first topic was the mode of action of kanamycin. Especially perplexing was the effect of dilutions of inocula of treated cells on viability. My own thought was simply that the toxic action of the kanamycin seems to resemble that of deprivation for streptomycin in streptomycin-dependent so called SD mutants. In the latter case, cultures may continue to grow for some length of time but yield primarily sterile cultures. This being the case, a moderately dense inoculum could give a turbid plate whereas a small inoculum would give no visible growth. This point seems to be already substantiated by what was said about the behavior of cultures in broth, although it is obvious that more work on the kinetics of growth and the viability remains to be done in this system. A rather fanciful suggestion in the light of this analogy was that conceivably streptomycin might antagonize the lethal effects of kanamycin in the same sense as, so to speak, it antagonizes the lethal effects of deprivation of streptomycin in an SD mutant. In order to bring this out most clearly, it may be necessary to test the effects of kanamycin and their antagonism by streptomycin in a culture which is already streptomycin resistant. I would further suggest the use of gradient plates so that variable concentrations of streptomycin could be used in such a task.

It appears also important to include a routine morphological examination of bacteria subject to antibiotic action as part of the study of any antibiotic. At the very least, it should be possible to determine whether lysis or the abnormal growth plays an obvious role. For this purpose, there is no better technique than observation with an oil chamber under phase contrast microscopy, and the set-up that I have which I find most workable is a zeiss phase contrast microscope arranged with long working distance condenser. The latter is most essential for any work with oil chambers. We also had occasion to discuss the simple technique of oil chamber observation and its very wide applicability.

The next topic that was under discussion or at least that I will put next in this summary was the mode of action of penicillin. Much of this is covered in the manuscript of which I have already mailed a copy. I am also going to send you a summary of the scheme that Strohminger has made for the successive addition of amino acid residues in the formation of wall polypeptides. I was pleased that you were quick to note the likely role of this system as a suitable target for chemotherapy, and nothing would delight me more than to see you take an active interest in it. For the immediate present, I would judge that diamino pimelic acid remains as the most readily accessible target in that system as we have yet to learn whether the conjugates of the amino acids with muramic acid and with uridine diphosphate can be biologically active. There would be very little point in trying to construct analogues if the original wild type compounds, so to speak,

are ineffective as growth factors in appropriate test systems. We have every hope of being able to establish this within a reasonable length of time and of course I will hasten to let you know the results. In view of the chemical complexities of the work, I think it would be best to put off doing very much with the uridine diphosphate conjugates until we have more information on their behavior as growth factors. One of the more exciting possibilities would be the possibility of using a microbiological system for the construction of even more toxic conjugates by deceiving the enzyme systems with analogue substituents. In this connection, I am reminded of a paper that has already appeared by Gots and co-author in the Journal of Bacteriology within the last year or two in which they demonstrated the formation of a rather specific toxin in bacteria treated with penicillin. It is hard to believe that that story is connected entirely with ours, and the things recently worked with might serve to be a useful indicator in further pilot experiments. I will annotate this feature on the notes to be sent on Strohminger's scheme.

This brings us to the question of analogues of DAP. We have discussed this before and there probably is not much to be added now. A priori, it would seem likely that the monosulphonic analogue would be the most likely bet, and I was not really at all surprised that the disulphonic analogue proved to be inactive. As we discussed, the point of using the monosulphonic analogue is that one functional group was unimpaired and available for incorporation into the growing polypeptide chain while the substitution of sulphonic or carboxyl might be expected to interfere with further incorporation. Of course, to be tested, it would be necessary to synthesize this compound. But it seems to me that would represent considerably less of a challenge than would the synthesis of some of the uridine derivatives that were discussed in another context. It might be helpful also if any literature that already existed on analogues of lysine were searched for possible precidents for DAP. In so many systems of wall formation, lysine and DAP seem to be homologous that a model of a DAP analogue might well be founded on an effective analogue for lysine. The special virtue of a DAP analogue would be the selectivity of its function since it would be much less likely to be toxic to a mamalian host. This, I think, represents another library project as I am unable off-hand to recall any examples of lysine antagonists. I am also sending you the scheme that Gilvarg has let me have for the biosynthesis of DAP which may afford some other suggestions for how to intrude on that pathway.

We come then to possible means of approaching virus chemotherapy. We do not have to go again over the questions as to whether an antiviral agent would be desirable. We can best judge the therapeutic usefulness of such an agent when we have one that is satisfactory in other respects, and my own supposition is that we would be happy indeed to have one. It was pointed out that an empirical search for antiviral agents has been going on for a long time, and that while some interesting effects have been turned up, nothing very useful has really come out of it. At the present stage of our knowledge, it seems to me that the one process of virus infection that we can most readily attack is that of the absorption of the virus to the host cell. The next most likely is the emergence of the virus from the infected cell to become infective virus, and finally the least hopeful is the chance of blocking the actual intracellular multiplication of the virus. The last I have put in this category because of the supposition that virus multiplication follows very closely the same basic pathways as the biosynthesis of normal host RNA. As we get more information about the process, however, we may expect to be able to do more about it even at that level. Given this point, the best system for the study of an inhibitor of the absorption is obviously the influenza virus, because here in the hemagglutination system we have a well worked-out model of the adsorption of the virus to host cells. The early work on this subject can only be described as brilliant, and if you have never done so, I strongly

recommend that you look at Hirst's first two papers on the subject. It would be hard for me to persuade myself that later work has been of equal quality although there has been a good deal of it and much insight in some of it. I am also sending you a copy of Burnet's bibliography with check marks placed against those papers that I consider most relevant to the immediate subject. I can only account for the muddiness of some of the work that has been done in this field by assuming that there does not always pertain a simple understanding of the relationships between substrates and inhibitors and the significance of the hemagglutination reaction and its inhibition in that light. So I am going to say a few things that perhaps restate the obvious.

This is at least my own schematization of the work that has been done on the reactions between mucoids and cell surfaces with influenza virus. On this scheme which follows Burnet closely, the surface of the virus particles is studded with a number of molecules of an enzyme formerly called the receptor destroying enzyme and which we might now call neuraminidase in the light of Gottschalk's work. The virus surface also contains a number of other substances many of them obviously of host origin, in view of their seriological reactivity with antibodies against uninfected host cells. Also present in this skin is a fair amount of the mucoid substance that is evidently the substrate of neuraminidase and which is a universal component of cell surfaces, at least in the respiratory epithelium. The reaction between a virus particle and the host cell consists in the first instance of the formation of an enzyme substrate complex between the exposed group of one of the neuraminidase spots and a mucoprotein molecule on the cell surface. For various reasons, it is necessary to assume that following the first contact, the virus is able to conjugate at a number of points, there being, perhaps, some correspondence in geometry between the distances of the neuraminidase spots from one another and of the corresponding associate molecule on the host membrane. The attachment at either end of a single virus particle with two cells is the basic reaction of hemagglutination. One reason for postulating a multi-point contact is that once a single virus particle attaches to a red blood cell it apparently "browses" over the surface until it finally destroys all of the receptor mucin molecules present on that surface. In order to account for the virus particles remaining attached to a single cell once it has made a given contact a plausable lower limit of three contacts is necessary. This is so that when one bond is broken, there will still be two left which serve to bind the virus particle to that same cell. If there were only one or zero left, there would be a high probability of desorption of the virus from that cell once the enzyme had reacted with the substrate. Therefore, the agglutination reaction is a measure of the attachment of the virus enzyme for the homologous substrate. The inhibition of the agglutination represents the competative combination of these soluble mucoid molecules with the enzyme spots of the virus. So long as a sufficient number of the enzyme spots are pre-occupied with attachments to soluble mucins, there are no spots available for attachment to fresh blood cells or other cell surfaces. The soluble mucins like the receptor materials on cell surfaces are, however, destroyed by the neuraminidase; and therefore are not effective as inhibitors for an indefinite period of time. Probably for this very reason they are not feasable as long term therapeutic agents although Fazekas has proposed that they do have some role in the course of an influenza infection. If, however, the mucoid substances either as free soluble mucins or as the material attached to cell surfaces are treated with periodic acid for a limited period of time, I must emphasize limited, they retain their ability to form enzyme substrate complexes but the alteration of the substrate by oxidation by periodate makes them resistent to enzymatic splitting. In short, they have become competative inhibitors. I am not at all sure that the various workers in

this field have fully understood the equivalence of inhibitors and substrates, the principal difference being in the rate at which the substrates are destroyed by the enzyme. In brief, the initial complexing reaction must be postulated to be very much the same, and for an effective long-term therapeutic inhibitor, we require a material that will not be destroyed by the enzyme with which it is reacting. We want it to react with that enzyme in order to prevent the virus particle from effectively reaching the cell surface. There is, for example, a paper by Bogoch in the December 1957 issue of Virology which has just appeared and I am not at all sure that he understands this intimate relationship between effective substrates and inhibitors. I would also add that prolonged treatment with periodate as had already been discovered by Hirst results in the destruction of the mucoids to the point where they do not react at all with the enzyme. In order to obtain the intermediate stage of alteration that we are most interested in, that is where the mucin retains its complexing specificity for the enzyme but has so been altered that it can no longer be split, it is necessary to have a carefully timed regime of treatment with periodate. These matters have been gone into by Burnet but have been largely ignored by workers outside the Australian school for reasons quite mysterious to me. In my opinion, a most rational approach to the search for an inhibitor of the influenza virus might be found by looking for a compound of relatively low molecular weight that has properties similar to that of periodic-treated mucin. The reasons for wanting low molecular weight are, of course, to insure greater diffusability, to be able to achieve higher molar concentrations, and to minimize the probability of allergic or immune reactions to the material. It seems to me that there is no more likely pathway to the finding of such a substance than the modification of existing materials either those known already to act as complexants, i.e., "inhibitors" for the influenza virus or those materials which contain neuraminic acid and might be expected to yield derivatives having such properties. This paper by Bogoch which I just mentioned is rather interesting because it does point to the existance of complexing substances of moderately low molecular weight as he has described, these being conjugates of neuraminic acid with galactose and other sugar moieties, but I think he has veered away from the main point of his own discussion. It is curious that even Gottschalk can report that neuramin lactose which is a substrate for neuraminidase is not an inhibitor for it. If we have any understanding at all of the relationship between inhibitor and substrate, this is merely a quantitative statement which reflects the very high rate of splitting of this particular material as compared to its rate of complex formation. One mustn't forget that there will be circumstances where even neuramin lactose will be an inhibitor namely where it is present in high molar concentration as compared to that of the substances with which it is competing for the enzyme surface. We have to admit the possibility that specificities other than that of the specific bond in the substrate contribute to the binding of the enzyme with the mucoid. However, from what we know of other enzyme substrate reactions, it is likely that the specific core of the substrate is the most important feature. Reading Bogoch on page 463 where he attributes what residual inhibitory activity he found of periodate-treated ganglioside to traces of unoxidized ganglioside, it is obvious that he does not understand what has been said before about the effect of limited treatment with periodate on other inhibitors. There is no reason why the first stages of such a program should not continue to exploit what has already been found with periodate, and, as I have already indicated, even this well-known effect has not been adequately explored with a view to therapeutic usefulness. Nor, so far as I know, has any effort been made to find split products of periodated mucins which might retain inhibitory activity and might have the advantages already indicated, nor has there been any attempt to see whether the very treatment of neuraminic acid or neuramin lactose as one of a number of

potential substrates of neuraminidase will result in low molecular weight inhibitors. Now, as far as I know, the only modifying reagent that has ever been tested for its effect on this system has been periodate. Fortunately, the first one to be tried already worked, and this is perhaps the reason for it, so there is a very wide open field for the consideration of other modifying reagents which may very well give the sorts of substances in which you are interested. At any rate, we have here a very simple in vitro system for the rational search for a specific antiviral agent. Of course, a wide variety of hemagglutinin inhibitors are known, most of which will turn out to be useless especially those of biological origin. This is so because we are in those instances trying to use hemagglutination as a rather poor model of the whole process of virus infection itself, whereas in the program that I am now espousing we are using hemagglutination as a specific index for a very particular type of activity that we are trying to influence. Once substantial progress has been made with this in vitro system, there will of course be the necessity of further in vivo tests for therapeutic activity. I would again suggest using a step-wise approach in which the de-embryonated egg is the best material for discernment of liminal therapeutic effects. In this system, even native mucin and, to a much larger extent, periodated mucin have measurable therapeutic activity, and to my mind, it is better to have this as a base line than to have no response whatsoever for compounds which are on the road to your aim.

Colominic acid, the mucopolysaccharide which Barry had isolated from E. coli strain K235, was mentioned as being a prolific source of neuraminic acid residues. Barry's latest analyses suggesting that colominic acid is in fact nothing but the polymer of acetyl neuraminic acid. It is, of course, worth testing this material directly as a possible complexant (I hesitate to say inhibitor because of the ambiguity of it) for the influenza virus. But even if it is not itself active, it may prove to be a convenient source either of neuraminic acid or of neuraminic acid derivatives of various kinds. These derivatives might be sorted into two types: those in which the mucopolysaccharide is in a sense hydrolized or alcoholized, etc., to give glycosides of various sorts conjugated to other materials. I might add at this point that an extremely useful reagent for further study of the neuraminidases would be the nitro phenol neuraminide, and it is even possible that such a reagent would have some therapeutic possibilities or be available for the construction of other conjugates. It would be useful if it served as a substrate of neuraminidase because of the new facility that it would add to the studies since this would be a chromogenic substrate comparable to nitro phenol beta-D-galactoside. If it is merely an inhibitor, it, of course, represents the sort of thing you are interested in, and I mention it as being a possibly interesting source of material because of the ease with which the nitro group could be reduced to amino and further substitutions made from that point of view. It might be something of a long shot, but I could imagine worse experiment than trying to fuse or otherwise react neuraminic acid with nitro phenol, I mean colominic acid with nitro phenol, to produce such a galactoside. The other type of derivative would be those in which there was substitution along the residue itself. It may be that this is a will-of-the-wisp and that colominic acid has no real structural relationship to the neuraminidase which are involved in the mucins although this might seem improbable. However, we do not know the specific bonds by which the colominic acid is polymerized.

Finally, we had a discussion on the more general aspects of serendipitous substitution which I feel possibly does not need to be elaborated again in a memorandum. The general issues and advantages of this approach were discussed, but they have already been presented to you in previous memoranda. I most earnestly hope that you are going to have an opportunity to explore this approach for which

a laboratory set up of your own, if it is to be realtively small and flexible, ought to afford the most congenial setting. If it really proves not possible for you to manage it in this way, then I hope some thought can be given to making other arrangements. For example, if necessary through your Japanese subsidiaries or what not, although naturally I would relish the opportunity of keeping in close-hand touch with it myself.

I will close with a reminder that I am expecting to receive from you, possibly already in the mail, some samples of novobiocin and of kanamycin, and also part of the bibliography on ascorbic acid bactericidal effect that has to do with the experimentally determined toxicity of ascorbic acid for bacteria and viruses. I will be sending you the further memoranda that I have already mentioned, and in addition a culture of E. coli strain K235 the source of colominic acid. I want to have a brief look at this myself because Goertl whom I saw in New York emphasized the colony variation that is found in this strain. The more opaque mucoid-looking colonies on nutrient agar being the ones which produce large amounts of neuraminic acid. In fact, perhaps the best way to produce the material in small amounts would be to make surface cultures of the organism on agar rather than precipitate material from broth as recommended in the original paper. If you do have occasion to prepare some colominic acid, I would be rather interested to have a small sample for some side tests to do myself, although possibly Jack Strohminger who is interested in the biosynthesis of colominic acid, as he has already been working on the biosynthesis of neuraminic acid, may be able to do that earlier.

If and when I get this back, it will help to serve as a reminder if I have left anything out. There was one other small point: I mentioned to you one other biological system, mainly the fact that strains like K235 carrying what I think is a cytoplasmic factor which is responsible for their ability to reproduce colicins. We may be doing some sort of screening ourselves to see if colicins can be removed by chemical reagents. In the present context of your work, it seems unlikely that you want to add another biological system to your screening procedures.

If I may record another impression that we have already discussed, it was that genetic analysis at this stage of your operations probably can not contribute a great deal to industrial improvement, that is, not without a considerable amount of foundational work still to be done. Partly for this reason, we did not spend a great deal of time in any further review of your basic antibacterial antibiotic program. Partly, too, this is because of my own conviction on the soundness of the serendipitous substitution scheme. However, I hope you will not hesitate to bring to my attention any features of your already established programs that might be interesting to look over. The suggestion was made that at least some of the bizarre compounds that streptomycetes make may be themselves precursors or modified precursors to their own walls. One of you at the meeting suggested that it might be worthwhile to see whether there was an appreciable level of streptomycin or other actinomycete-products in the walls of the organism that produced these things.

At this point, I just want to wish you and Pat and our co-workers at the laboratories the very best for the New Year, and have a good trip in Japan.

Yours sincerely,

Joshua
Joshua Lederberg

JL:ac